

K252a, a Potent Inhibitor of Protein Kinases, Promotes the Transition of *Dictyostelium* Cells from Growth to Differentiation

TAKESHI FURUKAWA and YASUO MAEDA¹

*Biological Institute, Faculty of Science, Tohoku University,
Aoba, Sendai 980, Japan*

ABSTRACT—Cell differentiation and proliferation are mutually exclusive processes in many cases. The transition of starving *Dictyostelium* cells from growth to differentiation phase has been shown to occur at a particular position (putative shift point; PS-point) in the cell cycle of *D. discoideum* Ax-2. The significance of phosphorylation states of proteins such as 101 kDa, 90 kDa, and 32 kDa phosphoproteins has been argued, particularly around the PS-point. In this study we examined effects of the protein kinase inhibitors and activators on the transition of Ax-2 cells from growth to differentiation. K252a, a potent inhibitor of protein kinases, inhibited growth possibly through the blockage of pinocytotic activity of cells, and promoted the progress of development after starvation when applied to Ax-2 cells at the growth phase. Such a K252a-effect was most pronouncedly exhibited on the cells located near the PS-point. Unexpectedly, however, the development of starved cells was found to be considerably delayed by staurosporine bearing a structural and functional resemblance to K252a when it was applied during the growth phase. Pulse-labelings of growing Ax-2 cells with inorganic ³²P (³²Pi) showed that K252a induces the disappearance of a 48 kDa phosphoprotein and the appearance of a 50 kDa phosphoprotein, specifically in the cells located around the PS-point. Phosphorylation of 32 kDa and 24 kDa proteins was also inhibited by K252a, but this inhibition was not necessarily specific to the K252a-treatment and occurred independently of the cell-cycle phases. The possible significance of these results is discussed in relation to a breakaway of cells from proliferation to differentiation at the PS-point.

INTRODUCTION

Cellular differentiation generally goes against growth, and the relationship between the two is a critical issue to be solved in developmental biology. In the life cycle of the cellular slime mold *Dictyostelium discoideum* axenic strain Ax-2, amoeboid cells grow and multiply by binary fission during the growth phase, as long as sufficient nutrients are supplied. This is followed by initiation of differentiation in response to nutritional deprivation to form multicellular structures that eventually develop to sorocarps consisting of a sorus (a mass of spores) and a supporting cellular stalk. Since the growth and differentiation phases are temporarily separated from each other and easily controlled by nutritional conditions, this organism is an excellent experimental system to analyze the mechanism of the transition from cell proliferation to differentiation at the cellular and molecular levels.

Ax-2 cells grow axenically with a doubling time of about 7.5 hr and have little or no G₁ phase [12, 21]. Commitment of Ax-2 cells to differentiation has been shown to occur depending upon the cell's position in the cell cycle at the onset of starvation [2, 6, 14, 16, 18, 22]. We have previously demonstrated using synchronized Ax-2 cells that cells progress through the cell cycle to a particular point (a putative shift point; PS-point) of mid-late G₂ phase, irrespective of the presence or absence of nutrients, and enter the differentiation

phase from this point in response to nutritional deprivation [14]. Thus the nutrient-responsive events occurring around the PS-point are of particularly importance for understanding the regulatory mechanisms of cell proliferation and differentiation. Recently, McPherson and Singleton [15] have reported a gene (V4) required for the transition from growth to differentiation in *D. discoideum* strain KAx-3; The V4 gene is expressed only during the growth phase, and the transformant into which the anti-sense RNA of the V4 gene was introduced shows normal growth but fails to express some differentiation-specific genes.

In general, it has been emphasized that phosphorylation and dephosphorylation of proteins play important roles in cell-cycle regulation and oncogenesis. For example, p105RB, the product of the retinoblastoma (RB) tumor suppressor gene, is maximally phosphorylated at the S phase of the cell cycle, while the RB protein is dephosphorylated at the G₀ and G₁ phases (reviewed in [4]). Dephosphorylation of the RB protein also occurs in cooperation with the induction of differentiation in several cell lines by phorbol ester- or retinoic acid-treatment [5]. Recently, we have proposed that in addition to dephosphorylation of a 32 kDa protein low phosphorylation levels of 101 kDa and 90 kDa proteins may be required for the transition of Ax-2 cells from growth to differentiation [1]. The experiments using the protein phosphatase inhibitors showed that the 101 kDa and 90 kDa proteins fail to be phosphorylated at the PS-point under the conditions of starvation [1]. On the other hand, Simon *et al.* [19] have found that intracellular 3',5'-cyclic adenosine monophosphate (cAMP) facilitates early develop-

Accepted October 28, 1993

Received September 13, 1993

¹ To whom correspondence should be addressed.

ment of Ax-2 cells, probably by activating the cAMP-dependent protein kinase (PKA). In the light of these findings, we paid attention to the involvements of protein kinases in the transition process of growing Ax-2 cells to the differentiation phase. For this, we examined pharmacologically how growth and differentiation are controlled by protein kinases, using a variety of inhibitors and activators of kinase activities. The results obtained showed that K252a, a potent inhibitor of protein kinases, inhibits growth and facilitates development when applied to Ax-2 cells growing around the PS-point. The autoradiographical analysis of ^{32}P -labeled proteins also demonstrated that a 48 kDa (or 50 kDa) phosphoprotein is affected specifically by K252a. The possible significance of our findings is discussed, with special emphasis on the mechanism of choice between growth and differentiation.

MATERIALS AND METHODS

Chemicals

K252a, staurosporine, and herbimycin A were purchased from Kyowa Medex (Tokyo, Japan) and dissolved in dimethyl sulfoxide (DMSO) at 1.0 mg/ml as the stock solutions. KT5720 and calphostin C purchased from Kyowa Medex were dissolved in DMSO at 4.0 mg/ml. 50 mM BAPTA/AM (Dojin Chem.) dissolved in DMSO was used as the stock solution. The stock solutions (200–720 mM) of W7 (N-(6-aminohexyl-5-chloro-1-naphthalene-sulfonamide; Sigma), 8-bromo 3',5'-cyclic adenosine monophosphate (8Br-cAMP), and 8-bromo 3',5'-cyclic guanosine monophosphate (8Br-cGMP) were prepared by dissolving them in ultra-pure water.

Growth conditions and synchronization of cell-cycle phase

Dictyostelium discoideum Ax-2 (clone 8A) was used in this study. Vegetative cells were grown axenically in HL-5 medium (Bacteriological peptone (Oxoid) 1.4 g, yeast extract (Oxoid) 0.7 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.13 g, KH_2PO_4 0.05 g in 100 ml of ultra-pure water) supplemented with 1.5% glucose [20]. Usually 10 ml of the cell suspension was shaken at 22°C at 150 rpm in a 200 ml-Erlenmeyer flask coated with Sigmacote (Sigma) on a gyratory shaker. Good synchrony was attained by a slight modification of the temperature shift method [12]: Ax-2 cells ($1\text{--}2 \times 10^6$ cells/ml) growing exponentially at 22.0°C were shifted to 9.7°C, shaken for 14.0 hr at 150 rpm, and then shifted again to 22.0°C. Under this condition, cell doubling occurred over about a 2-hr period after a lag phase of about a 1 hr; the PS-point being located near 7 hr after the shift-up from 9.7°C to 22.0°C.

Application of protein kinase inhibitors and activators to growing and starving cells

Non-synchronized or synchronized Ax-2 cells growing in HL-5 medium were treated with various concentrations of the drugs described in *Chemicals*, followed by monitor of change in the cell number. In another experiment, non-synchronized or synchronized Ax-2 cells were harvested, washed once by centrifugation (2,500 rpm, 75 sec) in 20 mM Na/K-phosphate buffer, pH 6.2 (PB) and resuspended in 1.0–1.5 ml of PB containing various concentrations of the drugs at a density of 1×10^7 cells/ml. The cell suspension was shaken at 22°C at 150 rpm in a 20 ml-Erlenmeyer flask coated with Sigmacote. After various periods of shaking, the cells were col-

lected by centrifugation, washed once in PB, and plated as droplets ($10 \mu\text{l}$) on non-nutrient agar (1.5% Agar Bacteriological, Oxoid) at 1×10^6 cells/cm². The preparations were incubated for various times at 22°C to allow development.

Pulse-labeling of cellular proteins with inorganic ^{32}P (^{32}P)

Non-synchronized or synchronized cell populations from various phases of the cell cycle (referred to as Tt cells), after the shift-up from 9.7°C to 22.0°C, were washed once in modified HL-5 (no addition of phosphate salts). The washed cells were suspended in 2 ml of modified HL-5 containing 0.3 mCi/ml of [^{32}P] ortho-phosphate (ICN, HCl-free) at a density of 2×10^6 cells/ml, and shaken for 3 hr at 22°C in a 20 ml-Erlenmeyer flask coated with Sigmacote.

SDS-PAGE and autoradiography

The ^{32}P -labeled cells (3.5×10^6 cells) were washed twice in ice-cold BSS (Bonner's standard salt solution; [3]) and dissolved in 90 μl of 10 mM Tris-HCl (pH 6.8) containing 2% SDS, 10 mM DTT, and 10% (V/V) glycerol, followed by heating in a boiling water-bath for 1 min. After cooling, 30 μl of the stock solution (0.5 M Tris-HCl, pH 7.0, 50 mM MgCl_2 , 1 mg/ml DNase I (Sigma), 0.5 mg/ml RNase A (Sigma)) was added to the sample, as described previously [1]. The samples (10–15 μl) were loaded on SDS-polyacrylamide gel (10%) and electrophoresed according to Laemmli [10]. Electrophoresis was carried out at 25 mA constant current until the dye (BPB) reached the bottom of the gel. Proteins in the gels were visualized by staining with silver [17]. For autoradiography, the electrophoregrams were fixed, dried, and exposed to a X-ray film (Amersham Hyperfilm-MP) with an intensifying screen (Kodak) for about 24 hr at -80°C .

Assay for pinocytosis

FITC-dextran FD-70S (Sigma) was used as a fluid-phase marker, as described previously [13]. Two ml of cell suspension ($2\text{--}3 \times 10^6$ cells/ml) growing exponentially in HL-5 was shaken in a 20 ml-Erlenmeyer flask for 1 hr at 22°C with and without either K252a (10 $\mu\text{g}/\text{ml}$ in 1% DMSO) or staurosporine (3 $\mu\text{g}/\text{ml}$ in 0.3% DMSO), followed by the addition of FITC-dextran at a final concentration of 3 mg/ml. After 1 hr of shaking at 22°C, pinocytosis was stopped by diluting a 2 ml-sample with 8 ml of ice-cold 20 mM PIPES buffer, pH 6.8. Cells were collected by centrifugation for 2 min at 1,500 rpm, washed, and resuspended in 2 ml of buffer. After centrifugation through 10% (W/W) sucrose for 3 min at 2,500 rpm, the cell pellet was resuspended in 3 ml of 50 mM Na_2HPO_4 . Cells were counted with a hemacytometer. Subsequently, cells were lysed by adding Triton X-100 (0.2% final concentration). The fluorescence intensity of the solution was measured with a fluorimeter (excitation wavelength, 470 nm; emission wavelength, 520 nm), and the amount pinocytosed was determined by comparison with a standard curve.

RESULTS

Effects of K252a and staurosporine on growth and pinocytotic activity

When K252a was applied to exponentially growing Ax-2 cells in HL-5, their growth was inhibited in a concentration-dependent manner, as shown in Fig. 1. In the presence of 5–15 $\mu\text{g}/\text{ml}$ K252a, growth of Ax-2 cells was completely inhibited at least during the first 5 hr of shake culture after the addition of K252a. Growth was also inhibited by 1 $\mu\text{g}/\text{ml}$ of

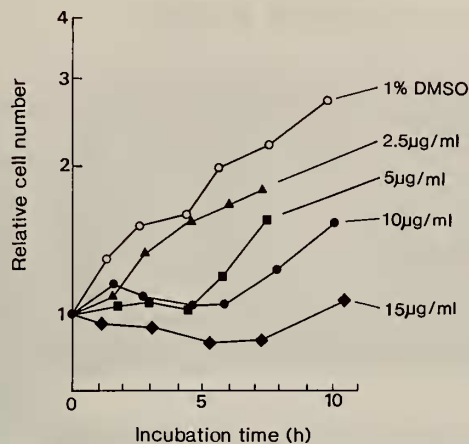


FIG. 1. Effect of K252a on growth of Ax-2 cells in axenic medium. Non-synchronized Ax-2 cells at the exponential growth phase were shaken in axenic growth medium (HL-5) containing the designated concentrations of K252a. Since 1% DMSO is contained in the test solution of 10 $\mu\text{g}/\text{ml}$ K252a, 1% DMSO (final conc.) was added to HL-5 as a control. In the presence of 15 $\mu\text{g}/\text{ml}$ K252a, cell proliferation is completely inhibited. A representative pattern of growth inhibition by K252a from one in three experiments is shown.

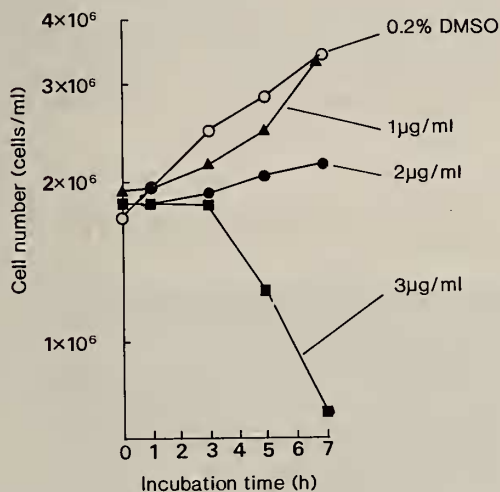


FIG. 2. Effect of staurosporine on growth of Ax-2 cells in axenic medium. Non-synchronized Ax-2 cells were shaken in HL-5 containing the designated concentrations of staurosporine. Since 0.2% DMSO is contained in the test solution of 2 $\mu\text{g}/\text{ml}$ staurosporine, 0.2% DMSO (final conc.) was added to HL-5 as a control. Cell proliferation is almost completely inhibited by 1–3 $\mu\text{g}/\text{ml}$ staurosporine at least during the first 3 hr after the drug application. A representative pattern of growth inhibition by staurosporine from one in three experiments is shown.

staurosporine during the first 3 hr of shake culture after its application, and almost completely in the presence of 2 $\mu\text{g}/\text{ml}$ (Fig. 2). A higher concentration (3 $\mu\text{g}/\text{ml}$) of staurosporine was toxic to the cells, thus resulting in a drastic decrease in cell number after 3 hr of shake culture. As shown in Fig. 1 and 2, growth of non-synchronized Ax-2 cells stopped immediately after the addition of K252a (5–15 $\mu\text{g}/\text{ml}$) or staurosporine (1–3 $\mu\text{g}/\text{ml}$). This seems to indicate that the

progression of cell-cycle phases is blocked by these drugs at the time-point of application.

Cellular drinking behavior (pinocytosis) is believed to be a characteristic property of many cells constituting a mechanism of nutrient uptake, and the pinocytotic activity of Ax-2 cells is high during the growth phase [11]. To light on the reason why K252a and staurosporine inhibited growth of Ax-2 cells, we examined their effects on pinocytotic activity using FITC-dextran as a fluid-phase marker. The results showed that pinocytotic activity is reduced by K252a or staurosporine in a concentration-dependent manner and is almost completely inhibited by 5–10 $\mu\text{g}/\text{ml}$ K252a or 1–3 $\mu\text{g}/\text{ml}$ staurosporine (Fig. 3). Thus it is most likely that growth inhibition by these drugs might be due to the failure of Ax-2 cells to pinocytose extracellular nutrients. Interestingly, however, the protein phosphatase inhibitors such as okadaic acid and calyculin A, which are known to inhibit completely growth of Ax-2 cells [1], scarcely inhibited the pinocytotic activity (data not shown).

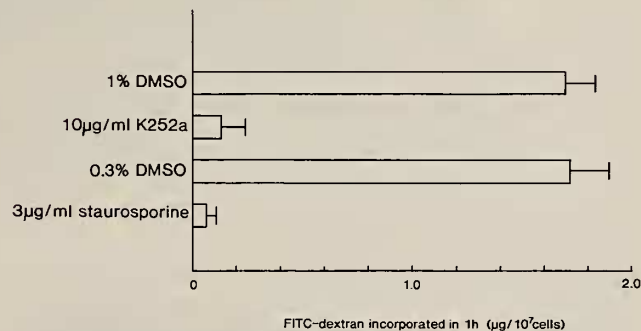


FIG. 3. Inhibition of the pinocytotic activity of Ax-2 cells by K252a or staurosporine. Pinocytotic activities were measured as described in Materials and Methods. 1% DMSO and 0.3% DMSO were used as controls of 10 $\mu\text{g}/\text{ml}$ K252a- and 3 $\mu\text{g}/\text{ml}$ staurosporine-treatments, respectively. Error bars represent standard deviations of three independent experiments.

Opposing effects of K252a and staurosporine on development

When Ax-2 cells at the growth phase were treated with 5 $\mu\text{g}/\text{ml}$ of K252a containing 0.5% DMSO for 3 hr, during which their growth was completely inhibited, washed free of the drug by repeated centrifugations in PB, and then allowed to develop on non-nutrient agar, the starved cells exhibited about 2–3 hr faster development as compared with a control containing 0.5% DMSO but not K252a (Fig. 4). In contrast to K252a, staurosporine (1.5 $\mu\text{g}/\text{ml}$) containing 0.15% DMSO caused delayed development of starved Ax-2 cells as compared with a control containing 0.15% DMSO but not staurosporine, when it was beforehand applied to growth-phase cells (Fig. 5). Although DMSO-treatment of Ax-2 cells at the growth phase caused somewhat delayed development by about 30 min as compared with another control without DMSO, 5 $\mu\text{g}/\text{ml}$ K252a (or 1.5 $\mu\text{g}/\text{ml}$ staurosporine) always elicited faster (or slower) development as compared with the control when the treated cells were starved.

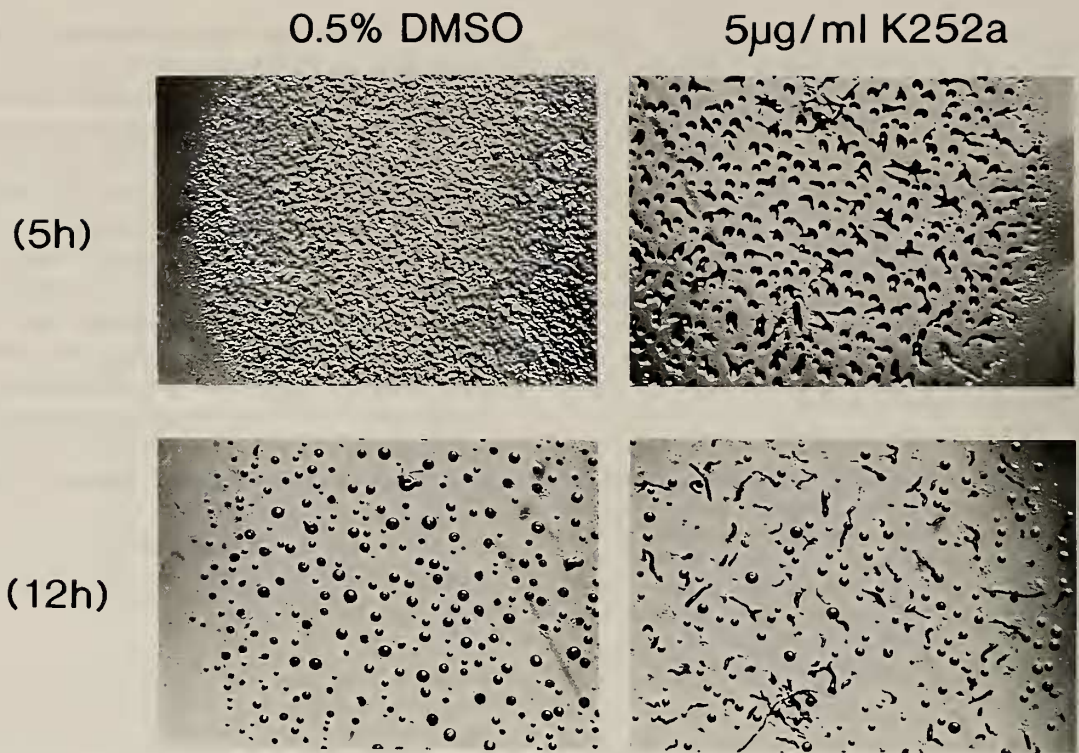


FIG. 4. K252a promotes the development of starved Ax-2 cells, if it was beforehand applied to growth-phase cells. Ax-2 cells at the exponential growth phase were treated with 5 $\mu\text{g}/\text{ml}$ K252a for 3 hr, washed, and then were allowed to develop on agar for the designated periods after starvation. As a control, cells were treated with 0.5% DMSO. The process of development including cell aggregation advances more rapidly in K252a-treated cells than in non-treated ones. A representative result from one in three experiments is shown.

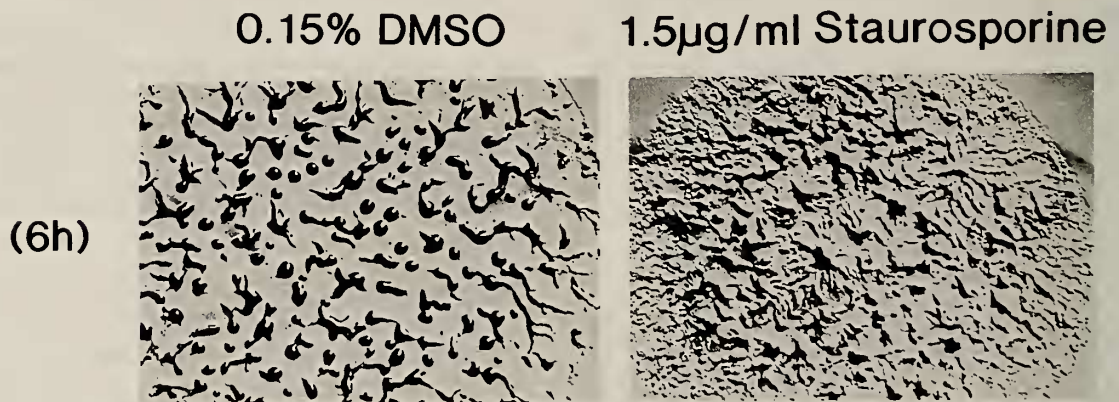


FIG. 5. Staurosporine inhibits the development of starved Ax-2 cells, if it was beforehand applied to growth-phase cells. Ax-2 cells at the exponential growth phase were treated with 1.5 $\mu\text{g}/\text{ml}$ staurosporine for 3 hr, washed, and then were allowed to develop on agar for 6 hr. The cells treated with 0.15% DMSO were used as a control. Development is considerably delayed by the staurosporine-treatment. A representative result from one in three experiments is shown.

The opposing effects of K252a and staurosporine on growing Ax-2 cells were found to be completely reversed if the drugs were applied to starving Ax-2 cells; t_0 cells (cells just after starvation) treated with 10 $\mu\text{g}/\text{ml}$ of K252a for 3 hr exhibited delayed development by about 1.5 hr as compared with a control. Meanwhile, $t_{0.5-3.0}$ cells (cells 0.5–3.0 hr after starvation) treated with 0.6 $\mu\text{g}/\text{ml}$ of staurosporine exhibited faster development (Fig. 6). Incidentally, the ap-

plication of staurosporine to t_0 cells had an inhibitory effect on their development, as the case for K252a (data not shown). Taken in conjunction, the effects of K252a and staurosporine on growing or starving Ax-2 cells are schematically shown in Fig. 7.

Cell-cycle dependency of K252a-effect

To examine whether or not the development-promotive

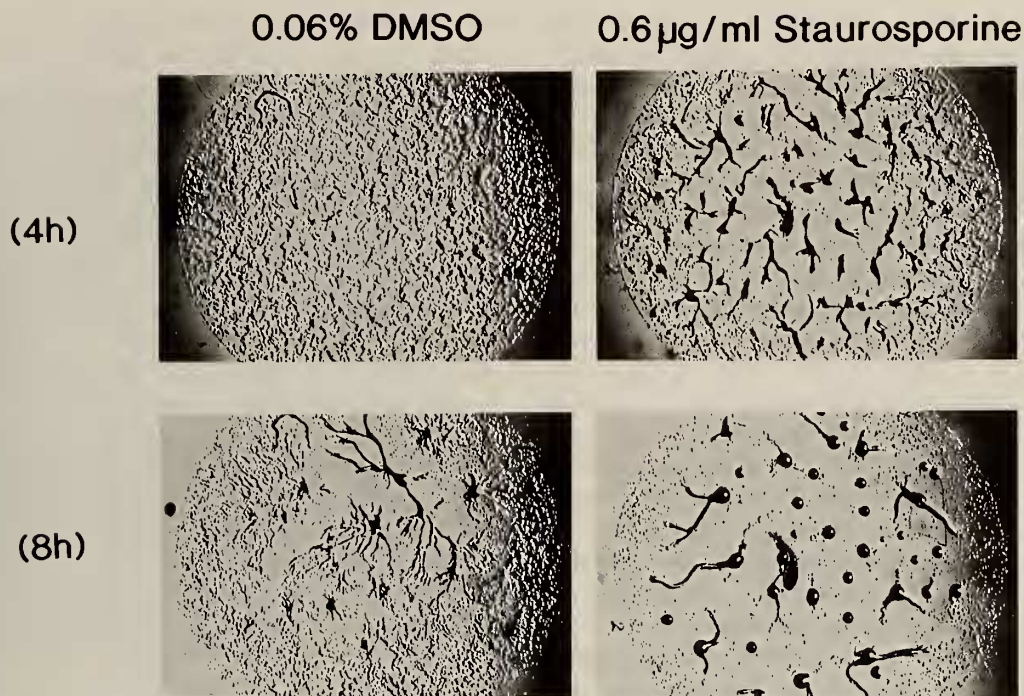


FIG. 6. Promotive effect of staurosporine on the development of starving cells. Ax-2 cells ($t_{2.5}$ -cells) 2.5 hr after starvation were treated with 0.6 $\mu\text{g/ml}$ staurosporine or 0.06% DMSO (as a control) for 2.5 hr, followed by washing and incubating on agar for the designated periods at 22.0°C. Cellular development is considerably promoted by the staurosporine-treatment. A representative result from one in three experiments is shown.

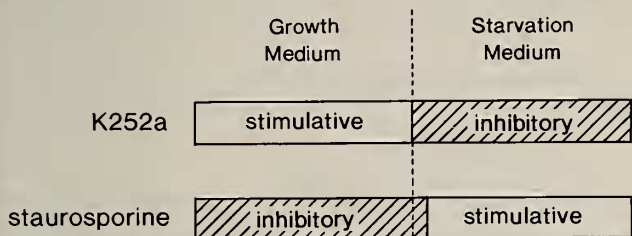


FIG. 7. A schematic diagram showing the opposing effects of K252a and staurosporine on growing and starving Ax-2 cells. Applications of K252a and staurosporine to cells either in growth medium or in starvation medium gave the contrasted effects on the development of starved cells, as indicated. See the text for details.

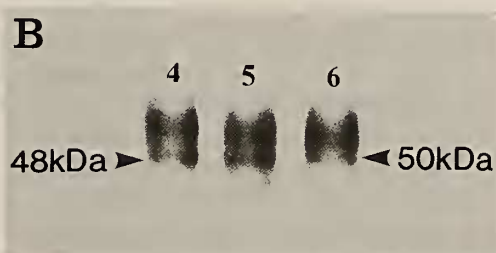
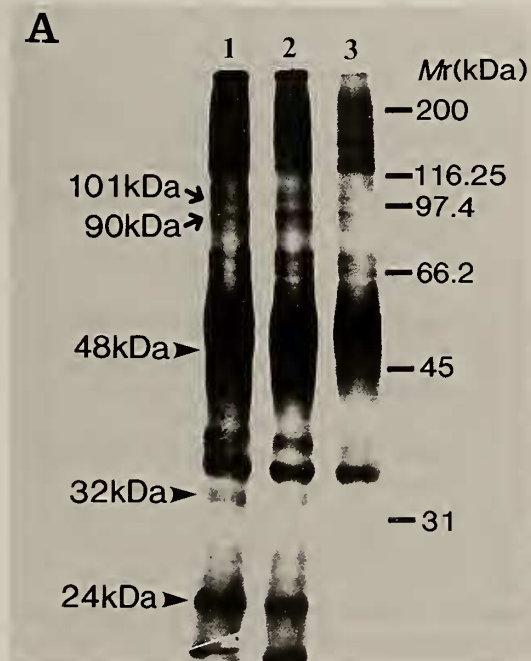


FIG. 8. Effects of K252a and staurosporine on protein phosphorylation. (A) Non-synchronized Ax-2 cells growing in HL-5 were labeled with ^{32}P i (0.3 mCi/ml) for 3 hr in the presence of 0.15% DMSO (lane 1), 1.5 $\mu\text{g/ml}$ staurosporine containing 0.15% DMSO (lane 2), and 10 $\mu\text{g/ml}$ K252a containing 1% DMSO (lane 3), and this was followed by autoradiography. Phosphorylation levels of 32 kDa and 24 kDa proteins are reduced particularly by the K252a-treatment. (B) An enlarged autoradiograph showing ^{32}P -labeling patterns around Mr of 50 kDa. Proteins labeled with ^{32}P in the presence of 1% DMSO (lane 4), 1.5 $\mu\text{g/ml}$ staurosporine containing 0.15% DMSO (lane 5), and 10 $\mu\text{g/ml}$ K252a containing 1% DMSO (lane 6). The disappearance of a 48 kDa phosphoprotein and the appearance of a 50 kDa phosphoprotein are observed on lane 6. Representative autoradiograms of the SDS-PAGE from one in three experiments are shown.

effect of K252a on growth-phase cells is manifested in a cell-cycle dependent manner, K252a was applied to synchronized cells from various phases of the cell cycle (referred to as Tt cells), after the temperature shift from 9.7°C to 22.0°C. K252a promoted markedly cellular development, particularly when applied to the cells at T6.5 (just before the PS-point). Application of K252a to T0.5-cells (just after the PS-point) caused less promotion of subsequent development after starvation. Therefore, it seems likely that the K252a-sensitive phase of the cell cycle may be located just before the PS-point.

Analysis of signal transducers affected by K252a

It is of interest to know which pathways of signal transduction are inhibited by K252a in growth-phase cells. For this, we applied specific inhibitors or activators as presented in Table 1 to Ax-2 cells growing in HL-5, followed by monitoring their effects on development as well as on growth. As summarized in Table 1, W7, the calmodulin inhibitor, blocked completely growth at a concentration of 30 µM, but the W7-treated cells exhibited normal development when they were starved by washings and allowed to develop on agar. Although 8Br-cAMP and 8Br-cGMP also inhibited growth at an extremely high concentration (40 mM), the development of the drug-treated cells after starvation was normal, as the case for W7-treatment. Other inhibitors including KT5720 (the specific inhibitor of PKA) had no

effects on growth and development, when separately applied within a range of concentrations indicated in Table 1.

Phosphoproteins affected specifically by K252a

To find out proteins whose phosphorylation is inhibited specifically by K252a, non-synchronized Ax-2 cells were labeled with ³²P_i for 3 hr of shake culture in HL-5 with or without 10 µg/ml K252a, followed by autoradiography. As a reference, another ³²P-labeling experiment was carried out in the presence of 1.5 µg/ml staurosporine, because it delayed the progression of development in contrast to K252a when applied to growth-phase cells, as previously described. From comparison of the ³²P-labeling patterns, K252a was found to induce the disappearance of a 48 kDa phosphoprotein and the appearance of a 50 kDa phosphoprotein. K252a also inhibited markedly phosphorylation of 32 kDa and 24 kDa proteins, though it reduced the total amount of ³²P-labeled proteins (Fig. 8). Phosphorylation of 32 kDa and 24 kDa proteins, however, was found to be moderately inhibited by staurosporine, thus indicating that the inhibition is not necessarily specific to K252a. In contrast, the change of a 48 kDa (or 50 kDa) phosphoprotein seemed to be a specific event induced by K252a (Fig. 8B). Considering from our previous findings [1], we expected that phosphorylation of 101 kDa and 90 kDa proteins would be reduced by K252a, but such reduction was not detected. Based on the development-promoting effect of K252a particularly on Ax-2

TABLE 1. Effects of various inhibitors and activators of protein kinases on growth and development of *Dictyostelium discoideum* cells

Drugs	Possible functions	Effects on	
		Growth	Development
K252a	inhibitor of protein kinases	I (5 µg/ml)	A (5 µg/ml)
Staurosporine	inhibitor of protein kinases	I (1.5 µg/ml)	I (0.75 µg/ml)
KT5720	specific inhibitor of PKA	N (5–17.5 µg/ml)	N (5–17.5 µg/ml)
Calphostin C	specific inhibitor of PKC	N (1–40 µg/ml)	N (1–10 µg/ml)
Herbimycin A	specific inhibitor of tyrosine kinase	N (1–40 µg/ml)	N (1–10 µg/ml)
W7	inhibitor of calmodulin	I (30 µM)	N (40–70 µM)
BAPTA/AM	Ca ²⁺ -chelator	N (10–500 µM)	
8Br-cAMP	membrane-permeable cAMP analogue	I (40 mM)	N (10–50 mM)
8Br-cGMP	membrane-permeable cGMP analogue	I (40 mM)	N (10–40 mM)

Non-synchronized Ax-2 cells growing in HL-5 were treated with the indicated drugs for 3–5 hr at 22.0°C, washed for starvation, and allowed to develop on non-nutrient agar. Effects of the drugs used are summarized as follows: A, promotion; I, inhibition; N, no effect. The value in the parenthesis indicates the optimum concentration of the drug for the case of A or I. In the case of N, the concentration-range examined is shown.

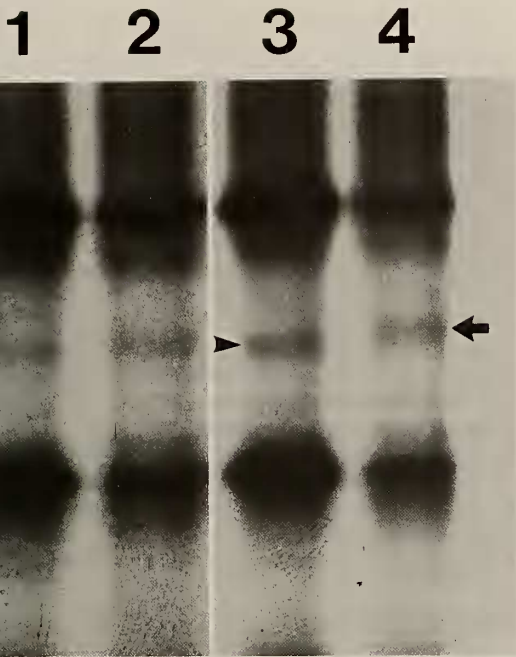


Fig. 9. Induction of the disappearance of a 48 kDa phosphoprotein and the appearance of a 50 kDa phosphoprotein by K252a in a cell-cycle dependent manner. Synchronized T0.5- and T6.5-cells were labeled with ^{32}P i (0.3 mCi/ml) for 3 hr in the presence of 1% DMSO (lane 1, T0.5-cells; lane 3, T6.5-cells) or 10 ug/ml K252a containing 1% DMSO (lane 2, T0.5-cells; lane 4, T6.5-cells), and this was followed by autoradiography. The change of a 48 kDa (arrowhead) or a 50 kDa (arrow) phosphoprotein seems to occur only in the K252a-treated T6.5-cells (lane 4). A representative autoradiogram of the SDS-PAGE from one in three experiments is shown.

cells growing around the PS-point, we examined effects of K252a on the phosphorylation patterns of proteins in synchronized cells. As a result, the disappearance of a 48 kDa phosphoprotein and the appearance of a 50 kDa phosphoprotein were found to occur in K252a-treated cells (T6.5-cells) just before the PS-point, but not in cells (T0.5-cells) just after the PS-point (Fig. 9). Incidentally, phosphorylation of the 32 kDa and 24 kDa proteins was inhibited by K252a, independently of the cell-cycle phases.

DISCUSSION

The present work was undertaken to examine the involvement of protein kinases in the transition of *D. discoideum* Ax-2 cells from growth to differentiation, using several inhibitors and activators of protein kinases. K252a, a potent inhibitor of protein kinases, promoted the progression of development, particularly when it was beforehand applied to Ax-2 cells growing just before the PS-point of the cell cycle. Unexpectedly, however, staurosporine that is believed to be similar in structure and function to K252a was found to delay the development of starved cells, thus being in striking contrast to K252a. In this connection, it is of interest to note that the sexual process (zygote formation) of

D. mucoroides-7 cells is promoted by K252a and inhibited by staurosporine [9]. Since K252a (or staurosporine) exerts an opposing effect on cellular development when applied under either the growth or starvation condition (Fig. 7), it is most likely that some of protein kinases are working differently depending upon the nutritional status of cells.

To specify the nature of kinase activities inhibited by K252a, we applied several specific inhibitors and activators of protein kinases, Ca^{2+} -chelater (BAPTA/AM), or calmodulin inhibitor (W7) to Ax-2 cells growing in HL-5, and this was followed by monitoring their subsequent development after starvation. Neither of them, however, had effects on the development of starved cells when they were separately applied to growth-phase cells (Table 1). Here, it is of interest to note that a combined application of KT5720 (a specific inhibitor of PKA) and W7 promotes the sexual development of *D. mucoroides-7* cells, as K252a does it, thus suggesting the involvements of PKA and calmodulin in zygote formation [9].

As presented here, phosphorylation of the 32 kDa and 24 kDa proteins was greatly inhibited by K252a. The 32 kDa protein has been previously shown to be perfectly dephosphorylated by serine/threonine-specific protein phosphatases in response to nutritional deprivation [1]. The low phosphorylation level of the 32 kDa protein in the presence of K252a might relate to the fact that K252a-treatment inhibits almost completely nutrient uptakes by pinocytosis, thus having Ax-2 cells starved. Dephosphorylation of the 24 kDa protein, however, was not induced by starvation in normal development. Recently, Hinze *et al.* [7] have shown by immunological analysis that a *Dictyostelium* protein of 32 kDa (p32) is equivalent to the *cdc2* and/or *cdk2* products of *Schizosaccharomyces pombe*, which contain the EGVP-STAIRESILLKE (PST) domain and bind $\text{p}13^{\text{suc}1}$ -agarose beads, a characteristic property of *cdc2*/*cdk2*-encoded protein kinases. It is of interest to know if the p32 is the same as the 32 kDa phosphoprotein reported by us.

We expected that phosphorylation of the 101 kDa and 90 kDa proteins would be inhibited by K252a, since their low phosphorylation levels might be associated with the entry of Ax-2 cells into differentiation from the PS-point. However, the phosphorylation level was not affected by K252a in the presence of rich nutrients around cells, suggesting that the 101 kDa and 90 kDa proteins might fail to be phosphorylated only after cells were starved around the PS-point. Interestingly, the disappearance of the 48 kDa phosphoprotein and the appearance of the 50 kDa phosphoprotein seemed to be specifically induced by K252a that promoted the progression of development, particularly around the PS-point. With respect to this phenomenon, one can imagine two possibilities; 1) a 48 kDa phosphoprotein exerts a phase-shift to 50 kDa, or 2) the disappearance of a 48 kDa phosphoprotein coincides with the appearance of another phosphoprotein of 50 kDa. Although it is presently unknown which is the case and remains to be elucidated, the observed change might be favorable to the transition of cells from growth to differentia-

tion at the PS-point. Hinze *et al.* [7] have reported that a *Dictyostelium* protein of 49 kDa (p49) is recognized by one of the PST antibodies, but not absorbed by p13^{suc1}-agarose, suggesting its partial resemblance to p34^{cdc2} in the PST domain. Based on the M_r and character, the p49 could be the same as the 48 kDa (or 50 kDa) phosphoprotein. We are now planning to isolate the gene encoding the 48 kDa or 50 kDa phosphoprotein to determine the chemical structure and also to analyze its function in cellular development.

ACKNOWLEDGMENTS

We wish to thank Dr. T. Takagi for his technical help with the phosphoamino acid analysis, Mr. S. Sato for assistance with the measurement of pinocytotic activity, and Mr. N. Iijima for his help with the autoradiographical analysis. This work was supported by a Grant-in-Aid (no. 04454018) from the Ministry of Education, Science and Culture of Japan.

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